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Analysis of protein chlorination by mass spectrometry

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ABSTRACT

Chlorination of tyrosine is a commonly known effect/consequence of myeloperoxidase activity at sites of inflammation, and detection of 3-chlorotyrosine has been used as biomarker for inflammatory diseases. However, few studies have addressed site specific chlorination in proteins, and no methods for large scale chloroproteomics studies have yet been published.

In this study, we present an optimized mass spectrometry based protocol to identify and quantify chlorinated peptides from single proteins modified by HOCl (100 and 500 μ M, within estimated pathophysiological levels), at a high level of sensitivity and accuracy. Particular emphasis was placed on 1) sensitive and precise detection of modification sites, 2) the avoidance of loss or artefactual creation of modifications, 3) accurate quantification of peptide abundance and reduction of missing values problem, 4) monitoring the dynamics of modification in samples exposed to different oxidant concentrations and 5) development of guidelines for verification of chlorination sites assignment.

A combination of an optimised sample preparation protocol, and improved data analysis approaches have allowed identification of 33 and 15 chlorination sites in laminin and fibronectin, respectively, reported in previous manuscripts [1,2]. The method was subsequently tested on murine basement membrane extract, which contains high levels of laminin in a complex mixture. Here, 10 of the major chlorination sites in laminin were recapitulated, highlighting the utility of the method in detecting damage in complex samples.

1. Introduction

A wide range of reactive species, including both free radicals and two-electron oxidants, are generated by cells during both normal physiological processes (e.g. enzyme reactions, electron leakage from electron transport chains) and as a result of exposure to external stimuli (e.g. radiation, UV light, metal ions, solvents, pollutants, etc) [3]. One major contributor to physiological oxidant generation is the innate immune system [3,4]. Activation of white blood cells, as a result of exposure to invading pathogens or other stimuli, results in the assembly of an NADPH oxidase (NOX2) complex on the plasma membrane, and the release of the heme enzyme myeloperoxidase (MPO) from storage granules in neutrophils and monocytes [4]. NOX2 employs intracellular NADPH to carry out univalent reduction of O_2 to superoxide radicals ($O_2^{\cdot-}$) [5,6]. The latter rapidly disproportionates (both spontaneously and via superoxide dismutase catalysed reactions) to give hydrogen

peroxide (H_2O_2) [7]. H_2O_2 is used by MPO, together with halide (Cl^- , Br^- , I^-) and other anions (SCN^- , NO_2^-) to generate a battery of highly reactive oxidants ($HOCl$, $HOBr$, HOI , $HOSCN$, NO_2^{\cdot}) that can induce oxidative damage to a wide range of biological targets [8]. As a result of the high physiological concentrations of Cl^- , $HOCl$ (hypochlorous acid) is the major oxidant produced by MPO under most conditions [8,9]. $HOCl$ reacts rapidly with multiple targets, but the high abundance of proteins in most biological systems, and the high rate constants for reaction of $HOCl$ with some protein side chains, results in protein damage being a predominant reaction [8]. The damage induced by $HOCl$ /MPO system, plays an important role in killing invading pathogens [4,10], but excessive or misplaced generation has been linked to a large number of human pathologies associated with acute or chronic inflammation, including cardiovascular diseases (e.g. atherosclerosis), rheumatoid arthritis, cystic fibrosis, sepsis, asthma, some forms of cancer, neurodegenerative conditions (e.g. Alzheimer's and Parkinson's

Abbreviations: 3-ClTyr, 3-chlorotyrosine; 3,5-Cl₂Tyr, 3,5-dichlorotyrosine; BME, basement membrane extracts; DTT, dithiothreitol; ECM, extracellular matrix; HCD, Higher energy collision dissociation; HOCl, the physiological mixture of hypochlorous acid and its anion $^{\cdot}OCl$; MS, mass spectrometry; RSO, relative site occupancy; SDC, sodium deoxycholate; TCA, trichloroacetic acid; TCEP, Tris(2-carboxyethyl)phosphine hydrochloride

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diseases) and kidney damage, amongst others [8,10,11].

Kinetic data indicate that the sulfur-containing amino acids (Cys, Met, cystine) are major targets, but damage can also occur on other side-chains including those of His, Trp, Lys and Tyr [12,13]. The products of these reactions have been mostly elucidated [14], but only a very limited number of these products are specific to HOCl/MPO-mediated reactions (i.e. the majority are also formed by other oxidants), hindering identification of the source(s) of biological damage and the quantitative importance of MPO-mediated damage [14]. Of the products known to arise from HOCl/MPO-mediated reactions, chlorination of Tyr residues to give 3-chlorotyrosine (3-ClTyr), and under harsh conditions the dichlorinated species 3,5-dichlorotyrosine (3,5-Cl₂Tyr), is the most well-established and specific biomarker of this oxidant system, though 3-ClTyr is quantitatively a minor product [8,13,14]. A number of studies have determined the total yield of 3-ClTyr in diseased versus normal tissue samples, and shown that elevated levels of 3-ClTyr are associated with disease severity in some cases [8,11]. The proteins on which 3-ClTyr is formed, the exact location(s) of this modified amino acid within protein structures, and the biological consequences of the formation of this species with regard to disease initiation, progression and prognosis, is however poorly understood.

As MPO is released both extracellularly and in to the phagolysosomal compartments of neutrophils, most of the HOCl formed is generated externally to cells [4]. Furthermore, MPO is a highly-cationic protein that is known to bind avidly with the negatively-charged macromolecules, such as the glycosaminoglycans (GAGs) present in the extracellular matrix (both hyaluronan and the GAG chains of proteoglycans) [15]. As a result of its high reactivity, which limits its capacity to diffuse significant distances from its site of generation, HOCl is likely to induce localized damage and particularly to extracellular matrix materials to which the enzyme is bound [16]. This hypothesis is supported by the detection of high yields of oxidized species on extracellular matrix components extracted from the artery wall (~70% of the total oxidized species detected in human atherosclerotic lesions) [17], the detection of modified epitopes on ECM proteins [18], and the observation that enzymatic removal of the GAG chains from perlecan decreases the extent of protein damage [18]. Damage to ECM materials may be quantitatively and functionally important, as these material are highly abundant in tissues, have relatively long half-lives (unlike many cell proteins) and are poorly protected against damage by the low level of extracellular defence and repair [19,20].

Quantitative analysis of the role of HOCl and MPO-derived oxidants in disease has been hindered by technical difficulties in identifying and quantifying site-specific 3-ClTyr formation. The low abundance of this product, and a lack of enrichment methods, makes it difficult to detect. Currently, a few studies have addressed the issue using mass spectrometry on single proteins [21–28] or simple mixtures [29] with two of these studies addressing the relative extent of modification [26,28].

In order to investigate the role of HOCl/MPO-mediated damage in chronic inflammatory diseases we have developed an optimised mass spectrometry based method, which had been used to analyse chlorination of laminin and fibronectin, two major ECM proteins and had been presented in two recent manuscripts [1,2]. Alterations to these proteins appears to play an important role in ongoing inflammation [19,30], but presents an analytical challenge for mass spectrometry based experiments, because they are large (fibronectin, 440 kDa; laminin, 850 kDa), often highly glycosylated, and contain a large number of disulphide bonds, which makes them difficult to digest and obtain full protein coverage by mass spectrometry. The method optimisation steps and observations that led us to the development of the current protocol are presented in this manuscript.

2. Materials and methods

2.1. Materials

Human plasma fibronectin was purchased from Corning and murine laminin-111 from Sigma Aldrich. Cultrex murine basement membrane extract (BME) with reduced growth factor was from Trevigen. Lysyl endopeptidase (Lys-C) was from Wako. Trypsin (sequencing grade) was from Sigma Aldrich. All other reagents were purchased from Sigma Aldrich. All solvents were MS grade.

2.2. Oxidation of purified proteins and protein extract

Human plasma fibronectin, murine laminin-111, or murine BME were suspended in 100 mM sodium phosphate buffer, pH 7.4, at a concentration of 1 µg µL⁻¹. HOCl was added at concentrations of 0, 100, and 500 µM and incubated for 1 h at 21 °C. The HOCl stock was prepared in 0.1 M NaOH buffer, pH 12, and the concentration determined spectrophotometrically using a molar extinction coefficient $\epsilon_{292} = 350 \text{ M}^{-1} \text{ cm}^{-1}$ [31].

2.3. Sample preparation for mass spectrometry

Briefly, single purified proteins (fibronectin or laminin) were treated with HOCl (100 and 500 µM). Any residual HOCl and phosphate buffer were removed using 10 kDa spin-filters (Amicon Ultra-0.5 Ultracel-10K, Merck Millipore) following the manufacturer's instructions. Proteins were denatured using 4 M urea and 1% SDC (sodium deoxycholate) in 50 mM TEAB (triethyl ammonium bicarbonate) buffer for 3 h or overnight. Laminin-111 was de-glycosylated using PNGase F. Protein digestion was performed in two-steps using Lys-C for 2 h in 4 M urea buffer and for 18 h using trypsin in 1 M urea buffer. The digestion temperature was kept at 30 °C to minimize protein carbamylation. The SDC detergent was removed from the peptide mixture using acidification and ethyl acetate phase transfer as described before [32]. Complete protocol is provided in [supplementary File S1](#).

Variations to the protocol were made when indicated and carried out as follows:

1. The on-filter clean-up step was replaced with TCA (trichloroacetic acid) and acetone precipitation. Samples were mixed with 10% TCA and acetone (99.9%) at 1:1:8 ratio of protein:TCA:acetone at –20 °C followed by 1 h incubation at –20 °C, then centrifuged at 18,000 × g for 15 mins at 4 °C. The resulting pellet was washed twice in ice-cold acetone, dried at 21 °C, and resuspended in denaturation buffer.
2. Reduction and alkylation of cysteine residues was carried out using:
 - a) 10 mM TCEP (Tris(2-carboxyethyl)phosphine hydrochloride) and 40 mM 2-chloroacetamide in 50 mM TEAB and 1% SDC buffer on the spin-filter device and incubated for 10 mins at 95 °C.
 - b) Denaturation in 8 M urea followed by spin-filter buffer exchange, reduction in 10 mM DTT (dithiothreitol) for 1 h at 56 °C and alkylation in 55 mM iodoacetamide for 30 min at 21 °C in 50 mM TEAB. Non-reduced controls were subjected to the same denaturation strategy. This alkylation step was omitted in the reduction-only protocol.

Excess reactants were removed by on-filter buffer exchange and digestion carried out with 1:50 (w:w) trypsin.

3. To test the role of protein solubilising agents 4 M urea and 1% SDC were replaced with either 8 M urea, 1% SDC, or RapiGest according to the manufacturer's manual. When no urea was included, the digestion temperature was increased to 37 °C. RapiGest was removed by acid precipitation using formic acid.

2.4. Mass spectrometry analysis

1 µg of peptide mixture was loaded onto a 2-column LC system. For purified protein analysis the precolumn (5 µm particle size, C18 fused silica beads, 4 cm × 100 µm ID) and analytical column (3 µm particle size C18 fused silica beads, 20 cm × 75 µm ID) were mounted onto an EASY nLC 1000 system (Thermo Fisher Scientific). Peptides were separated at a flow rate of 250 nL min⁻¹ using solvent A (0.1% formic acid) with 5–38% solvent B (90% acetonitrile and 10% aqueous formic acid at 0.1% v/v) over a 60 min linear gradient, followed by a 8 mins wash from 38 to 100% solvent B, and 5 min of 100% solvent B. Analysis of BME was performed using an EasySpray Column (2 µm particle size C18 fused silica beads, 50 cm × 75 µm ID; Thermo Fisher Scientific) mounted on a Dionex UltiMate 3000 RSLCnano System (Thermo Fisher Scientific), with the separation gradient increased to 120 min.

Eluting peptides were analysed on a QExactive HF (Thermo Fisher Scientific) in positive ion mode using data-dependant acquisition. Full scans of *m/z* 400–1400 was recorded with 120,000 resolution, with the top 12 most intense ions selected for HCD fragmentation at a normalised collision energy of 28. Blanks were run between each sample to monitor and prevent carry-over.

2.5. MS data analysis and verification of oxidation site assignment

Qualitative comparison of protocols and treatments was carried out by applying two different approaches of database searching: a) Proteome Discoverer 2.1 using the following search parameters: parent ion tolerance: 4 ppm; fragment ion tolerance: 0.1 Da; trypsin: 2 missed cleavages; fixed modifications: carbamidomethyl (at C) *or* none; variable modifications: addition of one Cl (at Y,W), addition of 2Cl (at Y,W), addition of a single oxygen atom (O at W,M,H,C), addition of two oxygens (2O at W,M,C), and addition of three oxygens (3O at C). For analysis of data obtained from pure proteins we have used isoform specific databases downloaded from Uniprot for human fibronectin (Uniprot Acc. No. [P02751](#), isoforms –1 to –17) and murine laminin (P19137, Q61001, P02469, Q61292, P02468, Q60675, Q61789, Q61789-2, Q61092, P97927, Q9R0B6, Q61087) combined with the MaxQuant common contaminants database. Analysis of BME was carried out using the Swiss Prot (*Mus musculus*) database (accessed April, 2017). b) The GPM online search was used for laminin-111 with multiple-round searches against the mouse (male) ENSEMBL database using the following search parameters: parent ion tolerance: 20 ppm; fragment ion tolerance: 0.4 Da; fixed modifications: carbamidomethyl (C) *or* none; potential modifications: round 1: O (M,W,C), Cl (Y); round 2: 2O (M,W,C), including common PTMs and unanticipated cleavage.

All modified peptides were validated manually by checking that the following criteria were met: 1) identification of unmodified peptide; 2) coverage of modification site by fragment ion series; 3) correct assignment of peaks in MSMS spectra (neutral losses: Met + O (–64), Met + 2O (–80), Cys + O (–50), Cys + 2O (–66), Cys + 3O (–82)); 4) similar fragmentation patterns between modified and unmodified peptide(s); 5) increased intensity of *m/z* +2 isotope of chlorinated peptides; 6) reproducibility between samples treated with different concentrations of HOCl.

2.6. Quantification of peptides and relative site occupancy

Quantification of peptide abundances was performed using Progenesis QI for proteomics software (Nonlinear Dynamics). Data files (.raw, Xcalibur) were imported into Progenesis QI, where extracted ion chromatograms (XIC) were aligned across runs and quantified by integration of precursor peak area. The aligned features were exported as mascot generic file format (.MGF) and the database search was carried out using the peptide identification approach a) described above. Identification data were imported into Progenesis QI and combined with the quantification data. Final peptide measurements were

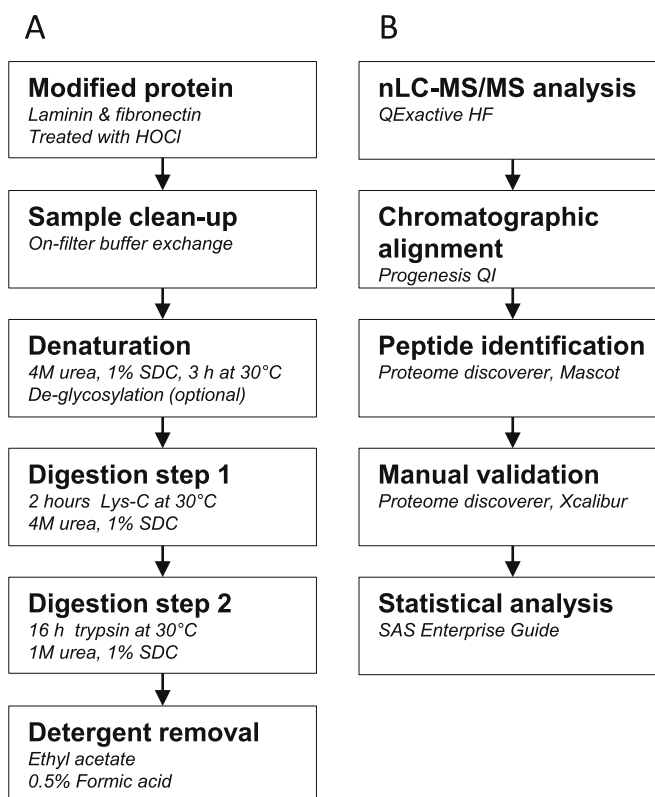


Fig. 1. Chlorination analysis workflow. (A) experimental steps of the optimised digestion protocol; (B) data analysis workflow for chlorinated peptide quantification.

exported as a .CSV file. All charge states of a peptide were pooled to eliminate bias in ionization. Relative modification site occupancy was calculated as the intensity ratio of the modified peptide against the sum of all peptide forms detected and is reported as a percentage, as described previously [26]. Changes in peptide abundance were evaluated by two-sided student's T-tests (with significance assumed at $p < 0.05$) using SAS Enterprise Guide 7.1 (SAS Institute Inc.).

3. Results

In chloroproteomics, there is a need for better detection methods for complex samples. We addressed this through consideration of sample preparation, peptide quantification and subsequent validation. We have developed an optimised sample preparation protocol for the preservation of protein chlorination and oxidation, minimising introduction of chemical artefacts and sample variation. The approach consists of the following steps: modification of selected protein using HOCl or MPO generated HOCl, sample clean-up to stop oxidation reaction, removal of excess oxidant and salts, denaturation, two-step digestion with LysC and trypsin endopeptidases, and detergent removal. The overall workflow is presented in Fig. 1A, with experimental details provided in [Supplementary File S1](#). This protocol is combined with MS based label-free peptide quantification based on chromatographic alignment, and manual validation of sequence and modification site assignments in peptide fragmentation spectra, Fig. 1B. The following sections describe key considerations in sample preparation and data analysis for studying labile oxidations and chlorination.

3.1. Gentle sample clean-up preserves native state of protein oxidation

Sample clean up and buffer exchange are commonly performed, prior to enzymatic digestion, to remove chemical contaminants and to provide optimal conditions for the activity of the enzymes. In protocol it

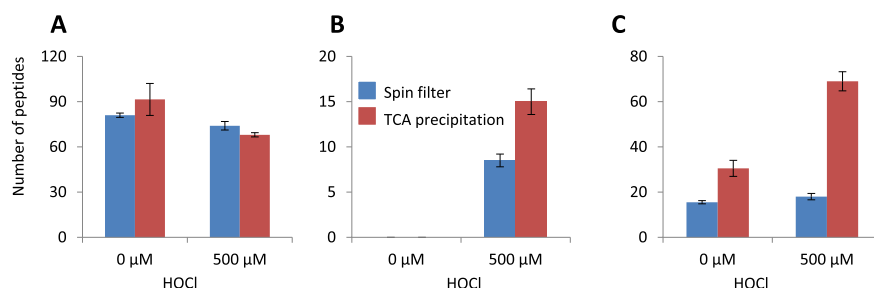


Fig. 2. Comparison of spin filter-based dialysis and TCA and acetone precipitation in the clean-up of modified protein after exposure to HOCl. Number of (A) unmodified, (B) chlorinated and (C) oxidized peptides, identified in human plasma fibronectin treated with 0 and 500 μ M HOCl. $n = 2$.

also serves to remove remaining oxidants. This step is commonly achieved by precipitation using TCA and acetone [33], and therefore treatment with a high concentration of organic solvent at low pH which may result in loss of modifications. As an alternative we have tested a spin-filter based clean-up method (Amicon® Ultra centrifugal filters) that deploys centrifugation-driven ultrafiltration through cellulose membranes, and does not require harsh chemicals used in other sample preparation workflows [34,35] and analysis of oxidized proteins [36,37]. Tests were carried out using fibronectin treated with 0 or 500 μ M HOCl. The two methods identified a similar number of unmodified peptides in treated and non-treated fibronectin (Fig. 2A, Supplementary Tables S1 and S2) indicating little effect on the overall performance of protein digestion and MS analysis. Both methods identified chlorinated peptides in the samples treated with 500 μ M HOCl, Fig. 2B, however more were identified in the protocol using TCA/acetone precipitation method (15 peptides) as compared to spin-filter dialysis (8 peptides). This could indicate either a better performance of the TCA/acetone method or introduction of uncontrolled artefacts. To evaluate the latter possibility we compared the number of oxidized peptides (O and 2O at M) in the untreated control samples processed using the two methods. We predicted that the method that resulted in the smaller number of modified peptides would be gentler, and least prone to induction of artifactual oxidations. The number of oxidations identified by TCA precipitation method was twice as high as for the spin filter method, Fig. 2C, with a similar trend observed for the samples exposed to 500 μ M HOCl. This indicates that the TCA precipitation protocol induced oxidations that were unrelated to the HOCl exposure, resulting in more complex analysis. The number of oxidised peptides detected using the spin filter method increased only slightly between control and HOCl treated samples (16 and 18 respectively). In subsequent experiments, published in Refs. [1,2], we have shown that many of the oxidation sites observed in control sample become quantitatively more oxidised upon treatment with HOCl as determined by relative site occupancy (RSO) factor.

3.2. Replacement of reduction and alkylation with urea and SDC detergent allows for effective protein digestion without losses of 3-ClTyr

Most sample preparation protocols for protein analysis using MS involve reduction of disulphide bridges followed by alkylation of free Cys [38] to improve digestion efficiency and avoid creation and re-formation of disulphide crosslinked peptides. However, when using DTT reduction in combination with iodoacetamide, we observed a loss of 3-ClTyr peptides from fibronectin (data not shown). To investigate the effect of alkylation on 3-ClTyr levels, we compared reduction and alkylation using TCEP and 2-chloroacetamide with reduction using DTT, and no reduction. Murine laminin-111 was used as a model protein as it contains laminin EGF-like (LE) domains rich in disulphide bonds. Our results show that alkylation, but not reduction, reduced the number of 3-ClTyr sites identified from HOCl treated samples, Fig. 3 and Supplementary Tables S3 and S4.

Peptide quantification revealed that DTT reduction affects peptide

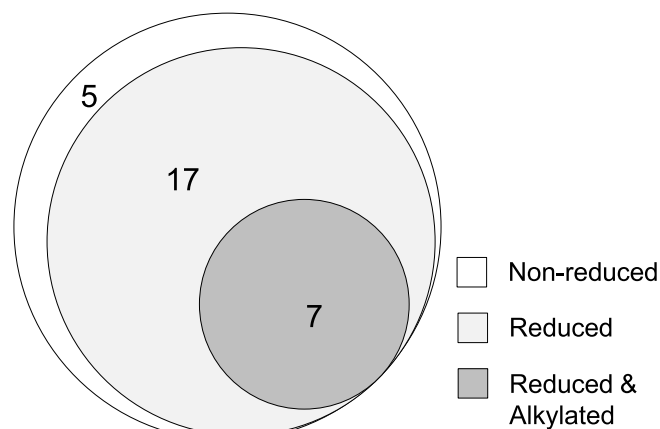


Fig. 3. Effect of reduction and alkylation steps on the number of identified 3-ClTyr sites in 1.18 μ M murine laminin-111 treated with 500 μ M HOCl. Protein digestion was performed omitting reduction and alkylation steps, with reduction step alone (10 mM DTT), and with reduction and alkylation (10 mM DTT and 55 mM iodoacetamide). Reported sites were identified by The GPM database search. The peptide lists are available in Supplementary Table S5.

levels both for oxidized and chlorinated peptides compared to no reduction, Fig. 4A. Most chloropeptides showed lower abundance, while Cys-containing peptides increased, Fig. 4B. Reduction with DTT led to increased sample variation (Supplementary Figs. 1A–B), with this largely attributed to Cys-containing peptides (Supplementary Figs. 1C–D). Supplementary Table S5 contains peptide quantification data.

To maximise digestion efficiency and protein coverage without a reduction and alkylation step, we tested the use of high concentrations of urea (up to 8 M), RapiGest [39] and SDC [40], and a combination of urea and SDC. A qualitative evaluation of the data obtained was made based on sequence coverage, the digestion efficiency evaluated by the proportion of missed cleavage sites, and the recovery of modified peptides, Table 1. Utilisation of 8 M urea led to the highest sequence coverage, however, it performed poorly in terms of digestion efficiency and recovery of chlorinated peptides. Both SDC and RapiGest resulted in ~2-fold increase, when compared to urea alone, in the number of chlorinated peptides, with RapiGest providing the highest digestion efficiency. A combination of 4 M urea with 1% SDC provided both high sequence coverage and recovery of modified peptides, with a digestion efficiency close to that of RapiGest, Table 1. Peptide quantification showed low reproducibility for urea, while the SDC-containing protocols provided high reproducibility (Supplementary Fig. S2). These data indicate that both SDC and RapiGest provide efficient and reproducible digestion, while urea increases the coverage, and that combining detergent and chaotropic denaturing agents can enhance protein digestion to provide high recovery and low sample variation. In the optimised protocol a combination of 4 M urea and 1% SDC, and two-step digestion using LysC followed by trypsin is employed (Supplementary File S1).

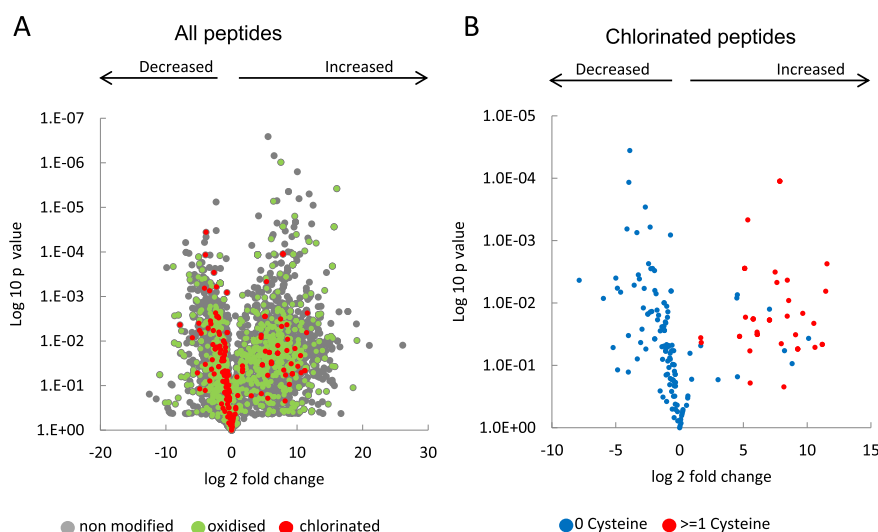


Fig. 4. Volcano plots illustrating changes in peptide intensities between reduced (DTT) and non-reduced laminin treated with 500 μ M HOCl. (A) all peptides identified; (B) peptides carrying at least one chlorinated residue. Increased and decreased refers to changes in intensity of the same peptide after reduction of the protein with DTT, $n = 3$.

Table 1

Protein coverage, digestion efficiency and number of modifications identified in fibronectin using different protein denaturation approaches.

Denaturants	Coverage [%] ^a	Digestion efficiency [%] ^b	3-ClTyr ^c	Oxidation (M, C, H) ^c
8 M Urea	55.6 \pm 3.6	89.5	11 \pm 1	14 \pm 3
1% SDC	44.2 \pm 3.7	91.8	21 \pm 2	13 \pm 3
4 M urea/1% SDC	51.0 \pm 0.1	96.5	21 \pm 2	14 \pm 1
RapiGest	46.6 \pm 3.6	98.1	21 \pm 3	17 \pm 3

^a Protein peptide coverage is calculated based on fibronectin protein samples treated with 0, 100, and 500 μ M HOCl (urea, SDC and SDC + urea treatments: $n = 9$, RapiGest treatments: $n = 6$).

^b Digestion efficiency is calculated by the number of peptide spectral matches (PSMs) matched to peptides without trypsin missed cleavage sites divided by the total number of PMSs per protein.

^c The number of modified amino acids found in fibronectin protein treated with 500 μ M HOCl (urea, SDC and SDC + urea treatments: $n = 9$, RapiGest treatments: $n = 6$). Data were obtained using Mascot search engine and Proteome Discoverer.

3.3. Identification of chlorination sites in fibronectin and laminin

We have reported that this optimised workflow can identify and quantify oxidation and chlorination sites in the extracellular matrix proteins fibronectin and laminin [1,2]. To evaluate the overall performance of the optimised method we have carried out meta-analysis of the previously published results [1,2]. Although digestion was carried out on non-reduced proteins, a high peptide coverage of 66% and 51% was obtained for fibronectin and laminin respectively summarized in Table 2. The regions of the proteins with poor coverage overlap strongly with regions rich in disulfide bonds, Fig. 5A. Treatment of both proteins with 0, 100 and 500 μ M HOCl allowed identification and quantification of 3-ClTyr and sites of Met and Trp oxidation, Fig. 5B. The overall extent of chlorination calculated as mean relative site occupancy of all chlorinated sites, was similar between the two proteins

Table 2

Summary of the number of unique modifications identified using the optimized sample preparation protocol extracted from Refs. [1,2]. Fibronectin, murine laminin-111, and murine basement membrane extract (BME) were treated with 500 μ M HOCl ($n = 3$).

Protein	3-ClTyr	3,5-Cl ₂ Tyr	Oxidation (M,C,W, H)	Sequence coverage [%]
Fibronectin	15	2	7	66%
Laminin-111 (chain α 1)	33	3	35	51%
Laminin from BME	11	5	23	29%

(~2%; Fig. 5C). Laminin exhibited higher levels of methionine oxidation (35% versus 23% in fibronectin; Fig. 5C). Meta-analysis of the results obtained from laminin and fibronectin [1,2] showed that the RSO of 3-ClTyr residues ranged from 7.7% to 0.02%, Fig. 6A. These low levels of 3-ClTyr residues present a technical challenge for studies aiming to identify chlorinated peptides in complex biological samples. As illustrated by peptide IYLYTLNDNAR in Fig. 6B the intensities of modified and non-modified forms can differ by factor 1000.

3.4. Validation of modification site assignment

All oxidative post-translational modifications identified in this study were verified by manual examination of MS/MS spectra, and it is proposed that modifications should only be considered valid if the following requirements are met:

The presence of fragment ions covering the modification site. The fragment ion series must match to the most intense ions in the spectrum. Possible neutral loss peaks including the addition should also be considered: Met + O (−64 Da), Met + 2O (−80 Da), Cys + O (−50 Da), Cys + 2O (−66 Da), Cys + 3O (−82 Da).

Presence of MS/MS spectra of the unmodified peptide and similar fragmentation patterns between modified and unmodified peptide(s). Examples of unmodified, 3-ClTyr and 3,5-Cl₂Tyr modified spectra are provided in Supplementary Fig. S3. The peptide contains two Tyr residues (Y₁₈₈₂ and Y₁₈₈₄) and the MS/MS spectrum shows the full y-ion fragment series with similar relative intensities for the unmodified and modified species, confirming that these originate from the same peptide. The mass shifts in the y₁₀ and b₂ fragment ions correspond to the addition of one and two Cl to Y₁₈₈₂, with no mass shifts observed for Y₁₈₈₄. This demonstrates that Y₁₈₈₂, but not Y₁₈₈₄, is a target for HOCl.

Characteristic isotopic patterns arising from the presence of the two stable isotopes ³⁵Cl and ³⁷Cl that contribute with 75.78% and 24.22% of abundances are detected, with these significantly different from the stable isotopic distribution of the main peptide components e.g. ¹²C (98.90%) and ¹³C (1.10%). The presence of Cl atom(s) in the peptide sequence can alter the typical isotopic peptide distribution, see

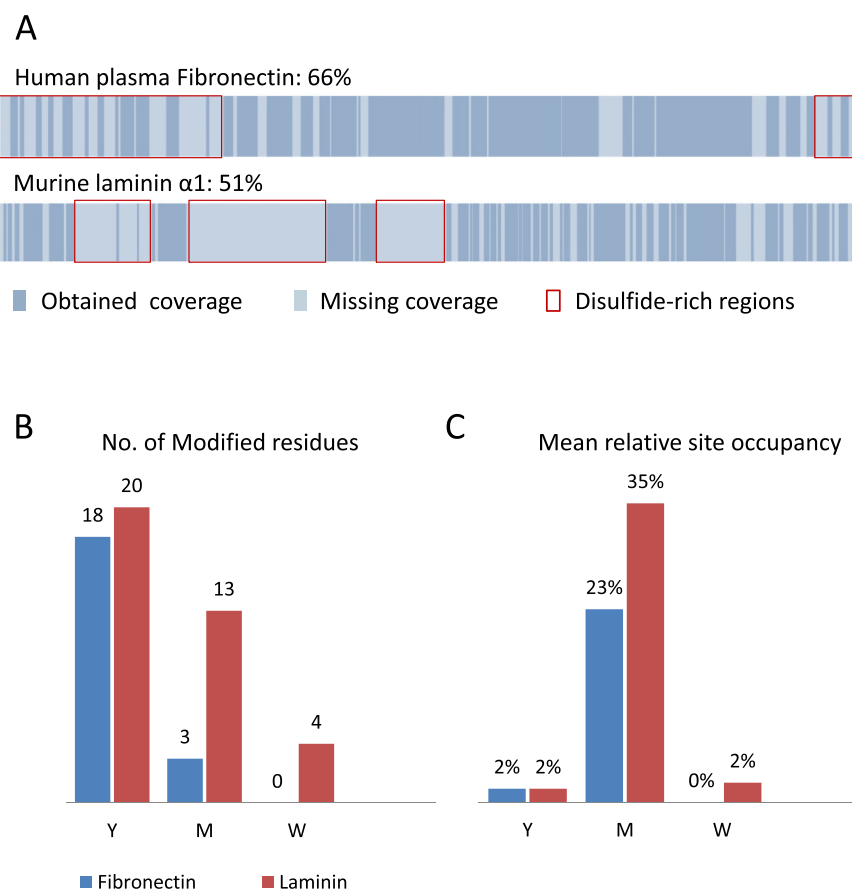


Fig. 5. (A) - protein map showing peptide sequence coverage from untreated controls. (B) – a total number of modified Tyr, Met, and Trp residues and (C) mean relative site occupancy RSO for modified Tyr, Met and Trp residues in human plasma fibronectin and murine laminin-111 treated with 500 μM HOCl.

Supplementary Figs. S4A–C. We have observed that the addition of a Cl atom changes the relative intensity of the 3rd and 4th isomer, which is particularly pronounced in peptides carrying more than one Cl atom, **Supplementary Fig. S4D**. Unfortunately, this is not always clear for all chlorinated peptides due to the length of the peptide, **Supplementary Fig. 4C**, and hence should not be the sole criteria used to detect chlorinated residues.

The reproducible presence of precursor across experimental replicates, and a low standard deviation should be considered as an important validation tool. Low levels of apparent chlorinated peptide may be present in controls due to “noise” or co-detection of near-isobaric ions. Inspection of extracted ion chromatograms can confirm the presence of the chlorinated peptide peak in treated samples and its absence in controls, **Supplementary Fig. S5**.

4. Discussion

We have developed a sample preparation protocol optimised for the analysis of chlorinated proteins, **Fig. 1**. This provides reproducible digestion under conditions that preserves both oxidized and chlorinated residues and is compatible with label free peptide quantification. By systematic evaluation of a number of sample preparation procedures we have identified key factors that affect the yield of chlorinated and oxidized products detected in MS-based experiments. Optimisation of these steps with alternative methods has allowed the detection of a large number of chlorinated and oxidized residues present in fibronectin (15 3-ClTyr and 7 oxidation sites), laminin (33 3-ClTyr and 35 oxidation sites) and basement membrane extracts (BME) (11 3-ClTyr and 23 oxidation sites) exposed to HOCl, as reported in Refs. [1,2], **Table 2**. The HOCl concentrations used in our studies (100–500 μM) are

believed to be pathophysiologically-relevant, on the basis of existing literature. Thus, it has been shown that activated human neutrophils at a concentration of 2×10^6 cells mL^{-1} (the concentration present in human blood) can generate between 150 and 200 μM HOCl over a period of 1 h at 37 °C using taurine as a trapping agent. It has also been reported that between 72% [41] and 90% [42] of the O_2 consumed during the respiratory burst of neutrophils is converted to HOCl, and that approximately 40% of the H_2O_2 generated is converted to HOCl [43]. Such data have been used to estimate the concentration of HOCl formed within neutrophil phagosomes as up to 0.3 M in the absence of targets, and 10 mM in the presence of competing targets (see **Fig. 3** in Ref. [42]). Other studies have reported experimental data of 17–170 μM HOCl (i.e. levels detected in the presence of native targets which will consume a considerable proportion of the HOCl) within neutrophil phagosomes [44]. Together these data indicate that concentrations of HOCl in the range 10–500 μM are of relevance at sites of inflammation, and in human pathology.

We have shown that TCA and acetone precipitation, which is widely used to remove small molecule contaminants from protein samples, leads to increased unspecific oxidation of control samples and increased chlorination of HOCl-treated samples, **Fig. 2**. These additional modifications may arise from the presence of residual oxidant and the effect of acetone on protein structure, with unfolding of proteins likely to expose residues that were not previously accessible to the oxidizing agent. As an alternative clean-up method, we propose spin-filter dialysis as reported initially by Manza et al. [34] and further developed by Wisniewski et al [45,46]. This procedure is currently the most widely used method for removal of detergents in proteomics experiments [47]. It efficiently removes detergents and other low molecular mass compounds and facilitates buffer exchange, without the need for organic

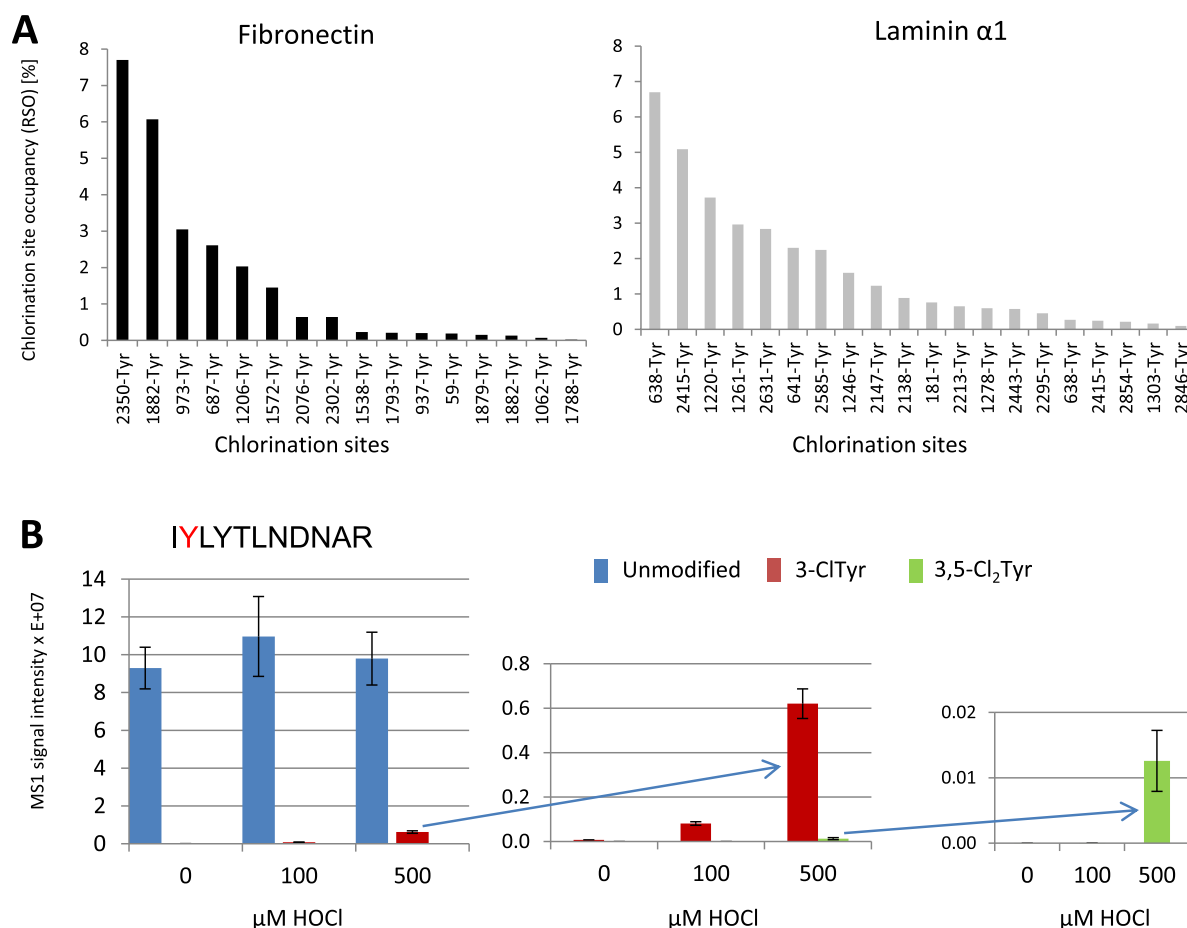


Fig. 6. (A) RSO of 3-ClTyr residues identified in human fibronectin and laminin $\alpha 1$ after treatment with 500 μM HOCl; (B) MS1 signal intensity of the peptide forms of fibronectin peptide $_{1881}\text{IYLYTLNDNAR}_{1891}$.

solvents, thus preserving the native protein structure and avoiding additional modifications, Fig. 2.

In mass spectrometry experiments chemical reduction of disulphide bonds followed by alkylation of the resulting free Cys residues is used to facilitate protein digestion and to prevent random formation of disulphide cross-linked peptides. Such peptides are difficult to fragment using typical fragmentation methods like low energy CID (collision induced dissociation) or HCD (higher-energy collisional dissociation), and the interpretation of the fragmentation spectra requires specialised software resulting in missing information in protein peptide coverage maps.

Unfortunately, the use of reducing and alkylating agents can affect experimental reproducibility by introducing undesirable side reactions [38,48]. Furthermore, it interferes with the analysis of certain protein oxidation products by reducing reversible modifications such as the mono-oxidation of Cys and Met residues, and converting peroxides (di-oxidations) to alcohols [49]. Two recent papers examining reduction and alkylation have reported adverse effects of such treatments [38,48], with both indicating an increased extent of off-target alkylation affecting the N-terminus, and Tyr, Thr, Ser, Lys, Asp, and Glu residues [38,48]. In our experiments we have observed a substantial loss of 3-ClTyr residues on treatment with 2-chloroacetamide and iodoacetamide. A similar observation was made by Chen and co-workers with iodoacetic acid [26]. The same group have reported that reduction and alkylation results in the loss of oxidized residues [50]. Tyr residues are known secondary targets of alkylation giving *O*-carboxamidomethyl Tyr [38,48,51], and we hypothesise that the chlorine substituent in 3-ClTyr may increase the rate of this process, but we have not detected such products in our samples. In our experiments protein reduction

alone caused a decrease in 3-ClTyr levels, but not to the levels induced by alkylation, Fig. 3. This is likely to be an ion suppression effect as consequence of the increased complexity contributed from Cys-containing peptides, present both as free thiols and randomly reformed disulphides as a consequence of the absence of alkylation, Fig. 4. The majority of previous published studies focusing on identification of 3-ClTyr residues have included reduction and alkylation steps in the sample preparation [21–25,27–29], and the current data suggest that such studies may therefore have underestimated the extent of modification. The use of DTT reduction alone also appears to increase sample complexity, and interferes with the detection of reversible oxidation products.

Successful and comprehensive identification of protein modification sites requires an efficient digestion process to give the highest possible protein peptide coverage maps. The necessity to omit reduction and alkylation prompted us to optimize protein digestion by examining the use of detergents and denaturation agents such as urea, SDC and Rapigest. Previous studies that have compared different protocols and solubilising agents have shown that in-solution digestion with SDC using spin-filter dialysis provides the highest reproducibility, high recovery and low sample variation [52,53]. Using a similar approach, we have compared SDC against urea, and the combination of urea and SDC, and found that the two agents combined enhance digestion yielding a high coverage while preserving the high reproducibility and peptide recovery, Table 1.

4.1. Challenges in quantification of oxidation and chlorination PTMs

Oxidation occurs readily at residues such as Met, during sample

preparation and electrospray ionisation [54,55], and therefore all sample handling may result in artefactual oxidation of susceptible residues. This is illustrated by the abundance of oxidized peptides in untreated samples, Fig. 2A. Significant oxidation of highly susceptible residues may therefore be present in control samples, and sensitive quantification is therefore necessary to distinguish between oxidant-induced and artefactual modification. In studies analysing labile and non-derivatized protein oxidations, stable isotope-based methods like for example TMT or iTRAQ are potentially problematic due to the additional handling steps that can increase variability. Label-free quantification presents a good alternative as it can be applied with minimal sample manipulation, and can be performed directly on bottom-up data [52].

Recent quantitative studies of 3-ClTyr formation have either used integration of the precursor peak area from selected reaction monitoring (SRM) with the abundance of the peptide carrying the modification calculated as a percentage of the total peptide [23,29], or by using a Native Peptide Reference (NPR) that determines the loss of the native peptide by selected ion monitoring (SIM) [22]. Of these two, SRM is the more reliable as it quantifies product formation in addition to monitoring the loss of native peptide. SIM and SRM are targeted methods, and thus highly selective and able to detect very low abundance species. However they are limited to a target list with a set number of ions, and requires experimental data to predict their retention time [56].

An interesting and biologically important facet in the quantification of PTMs by MS methodologies is the determination of each site's modification occupancy - the ratio (or percentage) of the modified and unmodified peptide form, as discussed in Refs. [57,58]. In this study we have used peptide intensities to calculate relative site occupancy (RSO), which takes in to account all observed products and calculates their percentage contribution, as described in Ref. [26]. This approach is based on the assumption that modified and non-modified peptides have similar ionisation properties. The advantage of such an approach is that it allows use of information obtained in shotgun analysis experiments where data acquisition is unbiased towards any selected type of peptides or PTMs, providing the possibility of looking for any type of modification retrospectively. Furthermore, it is compatible with labile and reactive modifications. Stable isotope-labelled internal standards, for both modified and non-modified peptides, would provide more exact data, but such an approach is currently very costly, time consuming and technically challenging given the number of species detected. As such it might only be performed for selected modification sites of interest.

A widespread problem observed in label-free MS data are missing values [59]. Due to sample complexity, the random (shot-gun) selection of ions for MS/MS fragmentation, the speed of the MS instrument, and its limit of detection, ions from low abundance peptides or peptides that are subjected to ion suppression are not fragmented and therefore MS/MS identification information is missing in some samples [60]. Different software developments have approached this problem and many studies have shown that Progenesis QI for Proteomics (Nonlinear Dynamics) performs best in limiting the number of missing values and providing comprehensive quantitative data from label-free experiments [60,61]. Progenesis QI performs chromatographic alignment between runs followed by peak picking with co-detection across all runs prior to database searching. In this manner, identification in one run is inferred to the corresponding extracted ion chromatograms (XIC) of other runs, thereby surpassing the issue of missing values [62]. Progenesis QI has recently been used to study chlorination in a single protein [63].

4.2. Evaluation of method performance using laminin, fibronectin and basement membrane extracts

We have detected and quantified 15 and 33 chlorination targets on Tyr residues in fibronectin and laminin, respectively [1,2]. This

approach has also been applied to BME, which contains laminin as a major constituent, with this resulting in the detection of 11 chlorination targets on laminin-111 in this mixture [2], demonstrating that the method can also detect modifications in complex samples. There are, to our knowledge, no studies on site-specific chlorination of ECM proteins in the literature. The largest chloroproteomics study reported to date identified a total of 14 chlorination targets in a mixture of nine proteins [29], making our studies the most comprehensive thus far. A major drawback of the current approach is the substantial loss of coverage of disulphide-rich proteins. This obstacle may be overcome by analysing disulphide-crosslinked peptides using dedicated approaches and software. It also requires that protein digestion is carried out at pH 6.5 to avoid disulphide scrambling [64,65], and may require further optimisation. Using the dedicated software tool MassAI [66] we provide proof-of-concept that 20–25% of the missing coverage in laminin-111 can be recovered by a Cys-Cys crosslink database search from our current sample preparation and MS strategy, Supplementary Fig. S6.

5. Conclusions

We have shown that adaptation of previous sample preparation strategies, can provide high coverage and reproducible data for large, disulphide-rich proteins without reduction and alkylation. Using label-free quantification at a peptide level, in shotgun MS of single proteins, chlorination can be detected down to a RSO level of ~0.15%. This method has also been shown to be effective for complex BME samples and may therefore be directly applicable to biological and clinical samples. Furthermore, this method minimizes artefactual oxidation, facilitating quantification of modifications to readily-oxidized residues such as Met, His, Cys, and Trp.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.redox.2019.101236>.

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